

Molecular properties of *Bacillus thermoglucosidius* exo-oligo-1,6-glucosidase*

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Abstract A *p*-nitrophenyl- α -D-glucopyranoside-hydrolyzing exo-oligo-1,6-glucosidase [dextrin 6- α -glucanohydrolase, EC. 3. 2. 1. 10] homogeneously purified from an obligate thermophile *Bacillus thermoglucosidius* KP 1006 showed an ultraviolet spectrum at pH 6.8 with a maximum absorption at 280 nm (A_{280} ; molecular extinction coefficient, 72,000 M⁻¹.cm⁻¹) and a minimum absorption at 250 nm (A_{250}), the ratio of A_{280} against A_{250} being 2.60. Its molecular weight, Stoke's radius, and sedimentation coefficient in water at 20°C were estimated as 60,000, 33.4 Å, and 4.95S, respectively. The isoelectric point was determined as 4.3. The enzyme contained neither cysteine nor cystine, and consisted of a single polypeptide chain constructed from 27 isoleucines, 14 phenylalanines, 36 prolines, 35 leucines, 28 valines, 17 methionines, 19 alanines, 33 glycines, 38 tyrosines, 43 lysines, 26 arginines, 30 threonines, 23 serines, 9 histidines, 76 aspartic acids, 59 glutamic acids, 6 tryptophans, and 54 amido residues. Rabbit antiserum prepared against the enzyme could not react on *p*-nitrophenyl- α -D-glucopyranoside-hydrolyzing isomaltases from a mesophile *Bacillus cereus* ATCC 7064 on the quantitative precipitin tests and double immunodiffusion. This finding suggests that no common antigenic determinant group exists on the molecular surface between the thermophilic and the mesophilic enzyme.

Introduction

The oligo-1,6-glucosidase [dextrin 6- α -glucanohydrolase, EC. 3. 2. 1. 10] activity was found in mesophilic bacteria such as *Streptococcus mitis*¹⁻³⁾ and *Lactobacillus bifidus*⁴⁾, and in hog intestinal mucosa⁵⁻⁷⁾. However, all of these mesophile enzymes have never been purified homogeneously, nor elucidated on their molecular properties. Recently, a *p*-nitrophenyl- α -D-glucopyranoside-hydrolyzing α -glucosidase, synthesized by an obligate thermophile *Bacillus thermoglucosidius* KP 1006⁸⁾, has been purified to a homogeneous state, and established to be tightly associated with the oligo-1,6-glucosidase activity with exo-type action⁹⁾. One of the most outstanding characteristics of this enzyme is its extraordinarily high resistance

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against inactivation caused by heat or by denaturing reagents ethanol, urea and sodium dodecyl sulfate (SDS)¹⁰. Thermodynamic studies from our laboratory have demonstrated that the thermophile enzyme undergoes the thermal conformational changes around 40–43°C, 61–64°C, and 73°C, the respective points which localize near the cardinal temperatures of the cell growth, and that the enzyme conformations present below 40–43°C are more rigid than those existing above it¹¹. In the present paper, we provide the molecular properties of the thermophile enzyme, including its amino acid composition. Also, we succeed in preparing rabbit antiserum against *B. thermoglucosidius* oligo-1,6-glucosidase. An evidence is achieved that the thermophilic enzyme and *Bacillus cereus* ATCC 7064 isomaltase able to hydrolyze *p*-nitrophenyl- α -D-glucopyranoside do not bear any common antigenic determinant groups on their molecular surfaces.

Materials and Methods

Materials. Bio-gel P-150 was purchased from Bio-Rad Laboratories; Sephadex G-25 and blue dextran 2000 from Pharmacia; horse radish peroxidase, beef pancreatic α -chymotrypsinogen, rabbit muscle lactate dehydrogenase, and horse liver alcohol dehydrogenase from Miles Laboratories; horse heart cytochrome c and bovine serum albumin from Sigma Chemicals; human hemoglobin from ICN Pharmaceuticals; chicken egg albumin and *p*-nitrophenyl- α -D-glucopyranoside from Seikagaku-kogyo Co. Ltd.; Freund's complete adjuvant from Difco Laboratories; collodion bag from Sartorius Membrane Filter; Ampholine pH 3.5–10 from LKB Aminkemi; Amido black from E. Merck Darmstadt; and agarose, *N,N,N',N'*-tetramethylethylenediamine, *N,N'*-methylenebisacrylamide, acrylamide monomer, and riboflavin from Nakarai Chemicals.

Enzymes. An electrophoretically-homogeneous oligo-1,6-glucosidase of *B. thermoglucosidius* (specific activity 245 units/mg protein) was prepared as described previously⁹. This preparation was used throughout all experiments in the present study, except the immunological tests. A partially purified enzyme (specific activity 174 units/mg protein), utilized in the immunological reactions, was obtained after hydroxylapatite chromatography in the enzyme purification¹⁰. *B. cereus* isomaltases I and II (respective specific activities, 45 and 115 units/mg protein) were kindly supplied as the partially purified samples by Miss Ritsuko Tanaka in this Laboratory.

Assay. The enzyme activity was determined spectrophotometrically in the reaction mixture (1 ml) containing 33.3 mM potassium phosphate buffer (pH 6.8), 2 mM *p*-nitrophenyl- α -D-glucopyranoside, and enzyme¹⁰. Incubation was for 1 to 3 min at 60°C with *B. thermoglucosidius* oligo-1,6-glucosidase, and at 35°C with *B. cereus* isomaltases. One unit of enzyme activity was defined as the amount of enzyme needed for hydrolysis of 1 μ mol of the substrate/min under the conditions as described above.

Protein. The concentration of the purified oligo-1,6-glucosidase was assessed by the turbidimetric method of Robenbloom et al.¹² The molecular weight was assumed as 60,000.

Spectrophotometry. Ultraviolet (UV) spectra of the oligo-1,6-glucosidase (2.03 μ M, 123 μ g in 1 ml) were recorded in 0.1 N NaOH and in 0.15 M potassium phosphate buffer (pH 6.8) including 1 mM ethylenediaminetetraacetate (EDTA) (Buffer A) with a Shimadzu

double beam spectrophotometer UV-200. Absorbance measurements were made with a Hitachi 101 spectrophotometer.

Gel filtration. Analytical gel filtration of the oligo-1,6-glucosidase was carried out at 20°C on a Bio-gel P-150 column (1.5×99.6 cm) equilibrated with Buffer A. The enzyme (80 µg in 1 ml of Buffer A) was applied and eluted with the same buffer at a flow rate 2.4 ml/29 min. The molecular weight (M_r) and Stoke's radius (a) were estimated by the method of Andrews¹³⁾, and by the method of Laurent and Killander¹⁴⁾, respectively. The standards employed to calibrate the column were as follows: bovine serum albumin (M_r =68,000; a =36.4 Å); chicken egg albumin (45,000; 27.3 Å); horse radish peroxidase (44,000; 30.2 Å); beef pancreatic α -chymotrypsinogen (23,500; 22.6 Å); human hemoglobin (31.3 Å); and blue dextran.

Gel electrophoresis and gel electrofocusing. Gel electrofocusing was done to determine the isoelectric point (pI) of the oligo-1,6-glucosidase, according to the method of Wrigley¹⁵⁾. Two 7.3% (weight/volume) polyacrylamide gels (0.5×7.2 cm) each containing the enzyme (75 µg) and Ampholine carrier ampholytes to give a pH gradient of 3.5–10 were prepared, and the electrofocusing was conducted at 2 mA/gel for 3 hr at 4°C. The first gel was sliced into 2-mm segments, and these were finely ground, followed by being mixed with 1-ml portions of water. The pH and enzyme activity of each extract were measured. The second gel was washed thoroughly with 5% trichloroacetic acid, before subjected to protein staining with Amido black as described below. Acrylamide gel electrophoresis of the enzyme (20 µg) was carried out at 4°C and 2 mA/gel for 2 hr in a 7.5% gel column (0.5×5 cm), by the method of Davis¹⁶⁾. The gel was stained for 1 hr in 1% Amido black dissolved in 7% acetic acid, and then destained with 7% acetic acid.

Sucrose density gradient ultracentrifugation. Sedimentation coefficient in water at 20°C ($S_{20,w}$) of the oligo-1,6-glucosidase was estimated by ultracentrifugation in a sucrose gradient according to the method of Martin and Ames¹⁷⁾. The enzyme [11.1 µg in 0.1 ml of 50 mM potassium phosphate (pH 6.8; Buffer B)] together with the standards was centrifuged for 24 hr at 3°C and 36,000 rpm in a sucrose gradient 5–16% including Buffer B, with a Hitachi 65P ultracentrifuge and a swinging rotor RPS-50. The 50% sucrose was injected into the bottom of the centrifuge tube to push up the gradient solution, and the 50 fractions (each 3 drops, 120 µl) were collected through a fine pipe from the top of the tube at a flow rate 40 µl/sec. The sedimentation zones of the enzyme were determined by assaying the enzyme activity in the fractions. Rabbit muscle lactate dehydrogenase ($S_{20,w}$ =7.00S), horse liver alcohol dehydrogenase (4.82S), horse radish peroxidase (3.85S), beef pancreatic α -chymotrypsinogen (2.58S), and horse heart cytochrome c (2.00S) were used as the maker proteins.

Amino acid analysis. The oligo-1,6-glucosidase sample (1.6 ml, 1.49 mg protein) was desalted by gel filtration through a Sephadex G-25 column (1.9×28.3 cm) with water as a solvent (flow rate 5 ml/15 min). The protein fractions collected were concentrated to 0.75 ml by ultrafiltration with a collodion bag. This was mixed with an equal volume of 12 N HCl. Hydrolysis was performed in a sealed evacuated tube at 110°C for 24 hr. After the HCl had been removed by evaporation, the dry matters were taken in 0.7 ml of 0.01 N

NaOH, and exposed to air for 4 hr in order to oxidize cysteine to cysteic acid. The hydrolysate was acidified by the addition of 0.7 ml of 0.1 N HCl, prior to analysis on a Hitachi KLA-5 amino acid analyzer. The amounts of the standard amino acids used for the analysis were as follows: proline and cysteic acid, 0.1 μ mol each; other amino acids and ammonia, 0.05 μ mol each. Tryptophan was estimated spectrophotometrically by the method of Bencze and Schmid¹⁸.

Antiserum. The homogeneous oligo-1,6-glucosidase (0.25 ml, 0.23 mg protein) was mixed with an equal volume of Freund's complete adjuvant in a small tube, after which the resulting thick suspension was injected under the back skin of a white female rabbit (3 kg). The tube was washed with four 0.25-ml portions of water. Each washing was emulsified in the adjuvant prior to injections. Three weeks later, a same amount of the enzyme was injected without adjuvant. The rabbit was bled four days after the last shot. The blood was centrifuged at 4°C and 12,000 rpm for 30 min with a Tomy-seiko ES-18GL centrifuge. The supernatant serum was stored frozen at -20°C until used.

Immunology. An agarose plate for double immunodiffusion was prepared by placing on a rectangular slide glass (1.7 cm width, 7.7 cm length, and 1.2 mm thickness) a 1.6-ml amount of melted 1.5% agarose including 25 mM borate-HCl buffer (pH 8.0), 0.1% Na₃N. After the gel solidified, one center well and six outer wells (each 2 mm diameter) were made in the gel. The center well was filled with the antiserum (3 μ l) against the purified oligo-1,6-glucosidase, and the outer wells with the partially purified samples (each 3 μ l) of the glucosidase (0.31 unit), *B. cereus* isomaltases I (0.41 unit) and II (0.62 unit). These materials were allowed to diffuse at 20°C for 24 hr. The plate was washed by soaking it in 0.85% NaCl containing 0.1% Na₃N, and by keeping at 4°C overnight. The precipitin lines formed were photographed. The precipitin reaction was quantitatively analyzed by adding a 10- μ l amount of the enzyme (0.08 unit oligo-1,6-glucosidase, 0.26 unit isomaltases) to the 0.85% NaCl solutions (0.1 ml) containing various amounts of the antiserum. After incubation for 20 min at 23°C, the mixtures were centrifuged at 2,300 rpm with a Kokusan tube H-103 centrifuge, to remove the resulting precipitates. The supernatants were assayed for the enzyme activity. Parallel experiments were run with the serum taken from an unimmunized rabbit.

Results

Molecular properties of B. thermoglucosidius oligo-1,6-glucosidase

(a) **UV absorption spectra.** As shown in Fig. 1, the oligo-1,6-glucosidase had a simple UV spectrum at pH 6.8, with a single peak at 280 nm (A_{280} , absorbance at 280 nm) and a trough at 250 nm. The ratios of A_{280} against A_{250} , and of A_{280} against A_{260} were 2.60, and 1.77, respectively. The molar extinction coefficient at 280 nm was calculated as 72,000 M⁻¹. cm⁻¹, and A_{280} of the 1% enzyme solution as 12.0 cm⁻¹. The alkaline spectrum in 0.1 N NaOH exhibited two characteristic maxima at 284 and 290 nm, and two minima at 269 and 286 nm (Fig. 1).

(b) ***Mr* and *a*.** The *Mr* of the oligo-1,6-glucosidase, estimated by the gel filtration, was 60,000, which agrees with the value gained by the SDS-gel electrophoresis as reported

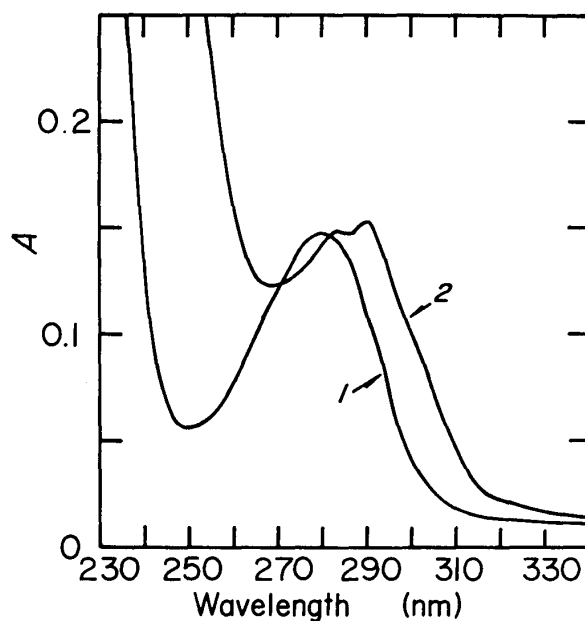


Fig. 1. UV spectra of oligo-1,6-glucosidase ($2.03 \mu\text{M}$) at pH 6.8 (spectrum 1) and in 0.1 N NaOH (spectrum 2). The spectra were obtained as described in Materials and Methods. A, absorbance.

previously⁹⁾. The estimated a value was 33.4 \AA .

(c) $S_{20,w}$. The $S_{20,w}$ was 4.95 S, achieved by the sucrose density gradient ultracentrifugation.

Table 1. Amino acid composition of oligo-1,6-glucosidase

Amino acids	Mole %	Residues *
Isoleucine	4.81	27
Phenylalanine	2.51	14
Proline	6.28	36
Leucine	6.24	35
Valine	4.91	28
Methionine	3.04	17
Alanine	3.42	19
Glycine	5.74	33
Cysteine + Cystine **	0 **	0 **
Tyrosine	6.72	38
Lysine	7.50	43
Arginine	4.58	26
Threonine	5.23	30
Serine	4.06	23
Histidine	1.59	9
Aspartic acid	13.5	76
Glutamic acid	10.3	59
Tryptophan ***	—	6 ***
Amide NH_2	9.57	54

* Calculated from the assumption that the enzyme contains 9 histidine residues.

** Determined as cysteic acid. *** Estimated spectrophotometrically¹⁸⁾.

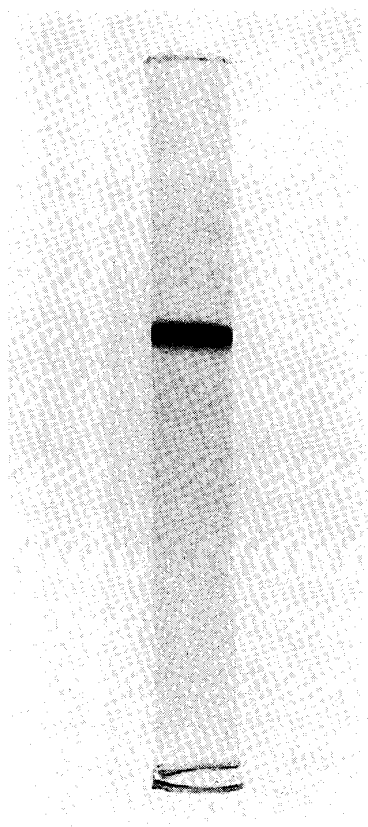


Fig. 2. Electrophoresis on polyacrylamide gel of oligo-1,6-glucosidase. The electrophoresis was conducted as described in Materials and Methods. The gel was stained with Amido black. The protein moved from the top to the bottom of the gel column in the direction from cathode to anode.

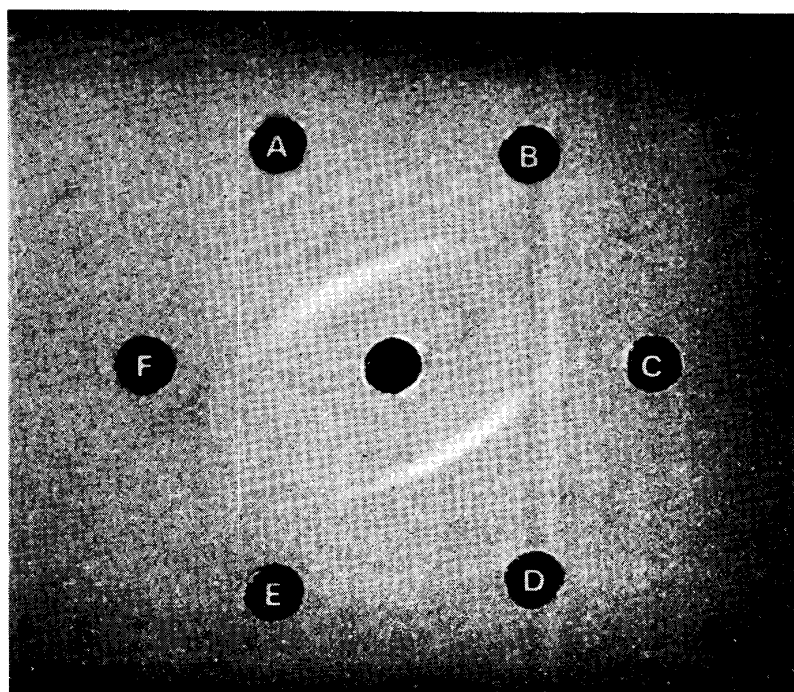


Fig. 3. Double immunodiffusion tests. The center well in agarose gel contained the rabbit antiserum prepared against the homogeneous oligo-1,6-glucosidase of *B. thermoglucosidius*, and the outer wells A and D contained the partially purified enzyme of the thermophile; the wells B and E contained *B. cereus* isomaltase I; and the wells C and F contained *B. cereus* isomaltase II. The antiserum and the enzymes were allowed to diffuse, as described in Materials and Methods.

(d) *pI*. It was found that the oligo-1,6-glucosidase behaved as a single protein band on the gel isoelectric focusing, the band which was completely coincident with the enzyme activity. The *pI* was determined as 4.3 from the pH-activity profile of the gel extracts.

(f) *Amino acid composition*. The amino acid constitution of the oligo-1,6-glucosidase is given in Table 1. It is characteristic that the enzyme contains neither cysteine nor cystine. Also, acidic amino acids glutamic acid and aspartic acid are relatively abundant.

(g) *Immunological properties*. Fig. 2 definitely shows that the oligo-1,6-glucosidase used as an antigen to immunize the rabbit was homogeneous on the polyacrylamide gel electrophoresis. No contamination of any other protein in the same sample had been proved in terms of the SDS-gel electrophoresis⁹⁾ and the isoelectric focusing. As shown in Fig. 3, in double immunodiffusion, a single sharp precipitin line was made between the rabbit antiserum against the homogeneous enzyme and its partially purified sample. This demonstrates that the enzyme employed for immunization was immunologically homogeneous. Also, Fig. 3 shows that the antiserum gave rise to no precipitin line with *B. cereus* isomaltases I nor II. As one can see in Fig. 4, the antiserum precipitated per μl 0.013 units of the thermophilic oligo-1,6-glucosidase. However, no precipitation was confirmed with the isomaltases (0.26 unit), using the antiserum up to 25 μl in 0.1 ml. It was found that the serum from an unimmunized rabbit failed to react on all above enzymes in the quantitative precipitin tests and double immunodiffusion.

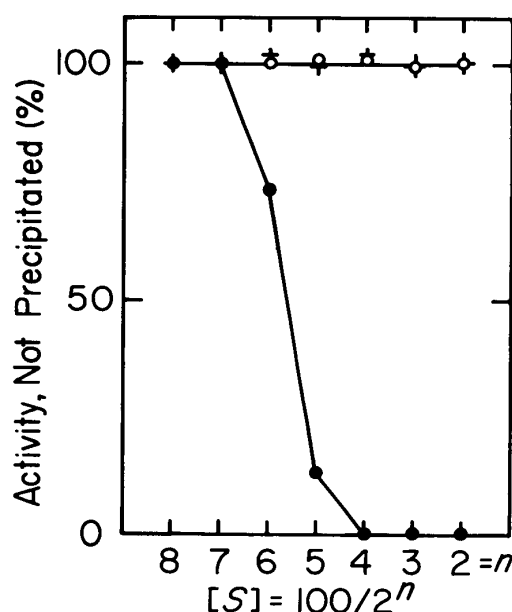


Fig. 4. Quantitative immunoprecipitation reaction of *B. thermoglucosidius* oligo-1,6-glucosidase (0.08 unit) (●), *B. cereus* isomaltases (0.26 unit) I (+) and II (○) with the rabbit antiserum against the thermophile enzyme. The experimental procedures were as described in Materials and Methods. The remaining enzyme activity in the supernatant of the reaction mixture was expressed as % against that in the absence of the antiserum. $[S]$, antiserum concentration (μl in 0.1 ml).

Discussion

B. thermoglucosidius oligo-1,6-glucosidase is a monomeric, nearly globular protein^{*1}, consisting of a single polypeptide chain. The UV spectrum of the enzyme is that of simple protein, indicative of absence of nucleotide or nucleic acid. Also, neither lipid nor polysaccharide is comprised as constituent in the enzyme molecule. It is a striking characteristic in the amino acid composition that it does not involve cysteine nor cystine (Table 1). This agrees with our earlier finding that the catalytic activity is not affected by thiol reagents¹⁰. Other prominent aspect in the composition is an abundance in the acidic residues glutamic and aspartic acids (23.8 mole %, compared with the basic residues lysine plus arginine, 12.1%), which is reflected in the acidic *pI* (=4.3). Such a relatively high content in glutamyl residues should be noticed in terms of their positive contribution towards thermal stabilization of many proteins²¹. It has been considered that hydrophobic interactions may be related to thermostability of protein²². Three parameters of relative hydrophobicity²³, $H\Phi_{ave}$, *NPS* and *p*, are computed as 1,050 cal/residue, 0.32 and 1.50, respectively, for the oligo-1,6-glucosidase from its amino acid composition. The $H\Phi_{ave}$ and *NPS* are not the especially big values, but within the most popular values ($H\Phi_{ave}$, 1,000–1,200 cal/residue; *NPS*, 0.30–0.38) found in many proteins including thermophilic proteins^{23,24}. The *p* is a far higher value in stead of a lower one (the most frequent *p*, 0.81–1.34^{23,24}).

As shown in Table 2, *B. cereus* isomaltases I and II^{*2} are not significantly different from *B. thermoglucosidius* oligo-1,6-glucosidase regarding their molecular and catalytic properties except thermal behaviors. This suggests the similarity in the gross structure of these enzymes, including the active site region. A number of studies have shown that thermophilic proteins are essentially homologous physicochemically to their individual mesophilic counterparts^{24,28}. A question that arises is whether there is a common structure on the molecular surface of *B. thermoglucosidius* oligo-1,6-glucosidase and *B. cereus* isomaltases. Such a homology would be possible, since these proteins of similar function are derived from the species in the same genus. However, the present study demonstrates that rabbit antiserum against the oligo-1,6-glucosidase does not act on either of the isomaltases (Figs. 3 and 4). This finding indicates that the thermophilic enzyme and each of the mesophilic enzymes does not bear any common antigenic determinant group on their molecular surfaces. At present, it is unknown whether such a difference on the surface is concerned with that in thermostability between the thermophilic enzyme and its mesophilic counterparts.

* ¹ The frictional ratio (f/f_0) was calculated as 1.29 by the method of Siegel and Monty¹⁹, using $M_r=60,000$ g, $a=33.4$ Å, and $v=0.728$ cm³g⁻¹ (partial specific volume from the amino acid composition²⁰).

* ² Isomaltase is other name of oligo-1,6-glucosidase, which has been often utilized when its substrate specificity is not completely established^{25,26}. *B. cereus* isomaltases I and II are acidic proteins like *B. thermoglucosidius* oligo-1,6-glucosidase, and distinguishable in their behaviors on the DEAE-cellulose column chromatography at pH 7.0: the enzymes I and II can be eluted from the column at the respective NaCl levels of 0.17–0.21 M and 0.32–0.45 M²⁷.

Table 2. Molecular and catalytic properties of *B. thermoglucosidius* oligo-1,6-glucosidase, and of *B. cereus* isomaltases I and II

Properties	Oligo-1,6-glucosidase *	Isomaltase I **	Isomaltase II **
<i>M_r</i> ***	60,000	60,000	57,500
<i>a</i> ***	33.4 Å	33.6 Å	32.5 Å
<i>S</i> _{20,w} ****	4.95S	5.00S	4.95S
pH optimum	5.0-6.0	6.7	6.7
Temp. optimum	75 °C	41 °C	45 °C
<i>K_m</i> for <i>p</i> NPG	0.24 mM	0.83 mM	0.60 mM
Isomaltose hydrolysis	Yes	Yes	Yes
Activity recovery on heating for 10 min at 60°C	100 %	0 %	0 %
Inhibition by <i>p</i> CMB or EDTA	No	No	No
Inhibition by Zn ⁺⁺	100 %	96 %	96 %

* 9, 10), and the present study. ** 27). *** Determined by gel filtration on Bio-gel P-150. **** Estimated by sucrose density gradient centrifugation. Abbreviations: temp., temperature; *p*NPG, *p*-nitrophenyl- α -D-glucopyranoside; *p*CMB, *p*-chloromercuribenzoate; EDTA, ethylenediaminetetraacetate.

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Bacillus thermoglucosidius の *exo-oligo-1,6-glucosidase* の分子的特性

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要旨：好熱菌 *Bacillus thermoglucosidius* KP 1006 の *p*-nitrophenyl- α -D-glucopyranoside を加水分解する *exo-oligo-1,6-glucosidase* の純化標品を用い、酵素の分子的特性と免疫学的性質を調べた。1) 分子的特性：a) pH 6.8での UV スペクトルの $\lambda_{\max}=280\text{nm}$, $\lambda_{\min}=250\text{nm}$, 280nmでの分子吸光係数 $72,000\text{M}^{-1}\text{cm}^{-1}$, 280nm の 260nm に対する吸光度比 1.77, 280nm の 250nm に対する吸光度比 2.60; b) Bio-gel P-150 のゲル濾過法で測定した分子量 60,000, Stoke's 半径 33.4 Å; c) 蔗糖密度配法で測定した $S_{20,w}=4.95\text{S}$; d) 等電点 4.3; e) アミノ酸組成, isoleucine 27残基, phenyl-alanine 14, proline 36, leucine 35, valine 28, methionine 17, alanine 19, glycine 33, tyrosine 38, lysine 43, arginine 26, threonine 30, serine 23, histidine 9, aspartic acid 76, glutamic acid 59, tryptophan 6, amide NH_2 54, cystine と cysteine 0。2) 免疫学的性質：oligo-1,6-glucosidase に対し調整されたウサギ抗血清は二重拡散法で部分純化酵素と単一沈降線を形成し、定量的沈降反応を起すが、中温菌 *Bacillus cereus* ATCC 7064 の対照酵素 isomaltases I と II とは全く反応しなかった。このことより好熱酵素と中温酵素には共通の抗原決定基が分子表面に存在しないことが判明した。